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Phorbol 12-Myristate 13-Acetate Protects Jurkat Cells from Methylglyoxal-Induced Apoptosis by Preventing c-Jun N-Terminal Kinase-Mediated Leakage of Cytochrome *c* in an Extracellular Signal-Regulated Kinase-Dependent Manner

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ABSTRACT

Methylglyoxal (MG) is an endogenous metabolite that increases in the blood and tissues of diabetic patients and is believed to be linked to the development of chronic complications of diabetes. We showed previously that Jurkat cells treated with MG rapidly undergo apoptosis via c-Jun N-terminal kinase (JNK) activation. In this study, we examined whether phorbol 12-myristate 13-acetate (PMA) can prevent MG-induced apoptosis in Jurkat cells. The results showed the following: 1) PMA can prevent MG-induced apoptosis; 2) triggering of this antiapoptotic signal depends on the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (ERK) pathway; 3) PMA inhibits MG-induced activation of caspase-3 and caspase-9, release of cytochrome *c*, and decline of mitochondrial membrane potential, but it does not affect MG-induced JNK activation; 4) the ERK pathway modulates outer mitochondrial membrane permeability and regulates the mitochondrial death machinery; and 5) activated ERK prevents JNK-induced leakage of cytochrome *c* from isolated mitochondria. Taken together, these results suggest that PMA-induced ERK activation can protect Jurkat cells from methylglyoxal-induced apoptosis and that activated ERK exerts its antiapoptotic effects on mitochondria by inhibiting activated JNK-induced permeabilization of the outer mitochondrial membrane.

Methylglyoxal (MG) is an endogenous metabolite that is produced from the process of degradation of triose phosphates, such as glucose or fructose, and is present commonly in mammalian cells (Chaplen et al., 1998; Thornalley et al., 1999). Under hyperglycemic conditions, it has been reported that the rate of MG formation increases because of elevated concentrations of precursors (Beisswenger et al., 1999, 2001). In fact, the serum concentration of MG increases by 5- to 6-fold in patients with insulin-dependent diabetes mellitus and by 2- to 3-fold in patients with non–insulin-dependent diabetic mellitus (Beisswenger et al., 1999, 2001). Because MG causes oxidative stress to cells that promote tissue damage (Baynes and Thorpe, 1999), it is believed to be a risk factor for the development of diabetic complications (Hammes et al., 1999).

We showed previously that Jurkat T lymphocytes exposed to MG rapidly undergo apoptosis in association with mitochondrial dysfunction and caspase activation (Du et al., 2000). Although the mechanisms by which MG triggers apoptotic events are not fully understood, our previous studies have suggested that intense activation of the JNK pathway triggered by MG contributes to mitochondrial dysfunction and subsequent cell death (Du et al., 2000, 2001). The JNK pathway is activated by diverse apoptosis-inducing stresses, including UV irradiation and exposure to hydrogen peroxide, heat, and osmotic shock. Recently, the JNK pathway has been demonstrated to be necessary for stress-induced cytochrome *c* release in embryonic fibroblasts (Tournier et al., 2000). More recently, it has been reported that JNK can directly induce cytochrome *c* release from mitochondria isolated from cardiac myocytes and brain cells (Aoki et al., 2002; Schroeter et al., 2003). These findings indicate that the JNK pathway plays an important role in the execution of oxidative stress-induced apoptosis.

ABBREVIATIONS: MG, methylglyoxal; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; MMP, mitochondrial membrane permeability; $\Delta\Psi m$, mitochondrial membrane potential; PKC, protein kinase C; MEK, mitogen-activated protein kinase kinase; DiOC6, 3,3-dihexyloxacarbocyanine iodide; z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; PD98059, 2′-amino-3′-methoxyflavone; FCS, fetal calf serum; PI, propidium iodide; PBS, phosphate-buffered saline; CHX, cycloheximide; Ras WT, wild-type Ras; Ras N17, dominant-negative Ras; NAC, *N*-acetylcysteine.

A number of studies have shown that ERK-mediated signals can abrogate Fas-induced death signals in T-cell lines and promote cell survival (Holmstrom et al., 2000; Herrant et al., 2002; Engedal and Blomhoff, 2003). According to those reports, in the Fas-mediated apoptotic process, activation of the ERK pathway can result in inhibition of the cleavage of caspase-8, which is required for the initiation of Fas-mediated apoptosis. This finding suggests that the ERK pathway plays an important role in the maintenance and promotion of survival of T cells under the condition of stimulation by Fas. Phorbol-12-myristate-13-acetate (PMA) activates members of the PKC family and transduces signals that regulate diverse biological functions. PMA can substitute for diacylglycerol, the endogenous activator of PKC, and stimulate the downstream signaling pathway of PKC. Various studies have suggested that PMA activates the Raf/MEK/ERK pathway efficiently via PKC activation in many cell types, including Jurkat cells (Li et al., 1999; Herrant et al., 2002).

In the present study, we tested the hypothesis that rapid activation of the ERK pathway after PMA treatment protects Jurkat cells from MG-induced apoptosis. We obtained evidence that PMA completely inhibits MG-induced apoptosis and that activation of the MEK/ERK pathway is an essential part of the mechanism of inhibition. The protection of cells against MG-induced apoptosis by PMA involves inhibition of mitochondrial depolarization and of caspase cleavage. However, PMA does not inhibit MG-induced activation of MKK4 and JNK. Notably, we found that conflicting signals transduced by ERK and JNK are integrated on mitochondria and that ERK prevents JNK-induced cytochrome *c* release by maintaining the outer mitochondrial membrane permeability. Finally, we obtained evidence that the MEK/ERK pathway plays an important role in the protection of murine splenocytes against MG-induced apoptosis. Our findings may provide a potential approach to the establishment of new therapies for tissue-degenerative disorders under hyperglycemic conditions.

Materials and Methods

Reagents and Antibodies. Methylglyoxal, PMA, digitonin, sodium orthovanadate, phenylmethylsulfonyl fluoride, 3,3-dihexyloxacarbocyanine iodide (DiOC6), z-VAD-fmk, aprotinin, valinomycin, and cycloheximide were purchased from Sigma-Aldrich (St. Louis, MO). Purified active ERK1, unactive ERK2, and active JNK1 were obtained from Upstate Biotechnology (Lake Placid, NY). Phospho-JNK, phospho-MEK, phospho-ERK antibodies, U0126, and PD98059 were from Cell Signaling Technology Inc. (Beverly, MA). Anti-JNK1, ERK1, Ras, Bcl-2, and caspase-3 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-cytochrome *c* antibody was from BD PharMingen (San Diego, CA).

Cell Lines and Culture Conditions. Jurkat human T-cell leukemia cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 1 mM glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Jurkat cells genetically deficient in caspase-8 were obtained from American Type Culture Collection (Manassas, VA). Jurkat cells stably expressing a wild-type Ras and dominant-negative Ras proteins were generated by electroporation under the condition of 270 V and 960 μF using a GenePulser II (Bio-Rad, Hercules, CA) with either pCMV-Ras or pCMV-RasN17 (BD Biosciences Clontech, Palo Alto, CA). Clones derived from each cell line were selected with 2 mg/ml G418. Jurkat cells were suspended at 2.5×10^5/ml in RPMI 1640 medium containing 5% FCS for in vitro culture study.

Splenocytes from C57BL/6 mice were prepared as described elsewhere (Bommireddy et al., 2003). Spleen was dissected out aseptically into a Petri dish containing RPMI 1640 medium and mechanically separated into individual cells. Contaminating red blood cells were depleted with red blood cell-lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 100 μM disodium EDTA) and washed twice with RPMI 1640 medium. Cells were resuspended at 1×10^6/ml in RPMI 1640 medium supplemented with 5% FCS for in vitro culture study.

Flow Cytometric Analysis. Apoptosis was judged by DNA fragmentation as described previously (Du et al., 2000). Stimulated cells were harvested and resuspended in propidium iodide (PI) buffer (0.1% Triton X-100, 0.1% trisodium citrate, and 50 μg/ml PI). After 15 min of incubation, the degree of DNA fragmentation was measured by an EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Nuclei to the "left" of the G_1 peak were considered apoptotic.

Loss of mitochondrial membrane potential was assessed using DiOC6 (Castedo et al., 2002). Stimulated cells were exposed to 40 nM DiOC6 and incubated for 15 min at 37°C. After washing with PBS twice, the cells were analyzed by a flow cytometer using an excitation light of 488 nm and an emission light of 525 nm (FL-1 channel).

Western Blotting. Western blotting was carried out as described previously (Du et al., 2000). In brief, cells were lysed with lysis buffer (0.5% Nonidet P-40, 20 mM Tris-HCl, pH 7.6, 0.15 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 20 μg/ml aprotinin, and 5 μg/ml leupeptin) and boiled for 3 min. The cell lysates were passed through a syringe with a 26-gauge needle and applied to SDS-polyacrylamide gels. The protein-transferred membrane was incubated with the first antibody and then with goat anti-rabbit IgG or sheep anti-mouse IgG conjugated to horseradish peroxidase, which was visualized using a Western blot chemical reagent. The specific immune complexes were detected using Western blot plus chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Boston, MA).

Preparation of Cytosolic Fraction for the Detection of Cytochrome *c*. Stimulated cells were harvested and washed with PBS. The pellet was washed once with buffer A (20 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM EGTA, 1 mM sodium orthovanadate, and 5 mM succinic acid) and resuspended in ice-cold buffer B (20 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM EGTA, 1 mM sodium orthovanadate, 5 mM succinic acid, and 40 μg/ml digitonin). After a 15-min incubation on ice with occasional shaking, the cells were centrifuged at 14,000*g* for 10 min. The supernatant was dissolved in SDS sample buffer, and cytochrome *c* in the supernatant was detected by Western blot analysis.

Separation of Mitochondrial and Cytosolic Fractions. Stimulated cells were washed once with PBS. The pellet was resuspended in a volume of buffer A and incubated for 15 min on ice. The cells were homogenized using a Dounce homogenizer and centrifuged at 1300*g* to remove the unbroken cells and nuclear fraction. The harvested supernatants were further centrifuged at 10,000*g* for 15 min. The resulting mitochondrial fraction (pellets) and the cytosolic fraction (supernatant) were dissolved in SDS sample buffer.

Cell-Free Assay of Mitochondria. To obtain mitochondria, Jurkat cells were homogenized with a Dounce homogenizer in buffer A. The homogenates were centrifuged at 1300*g* for 3 min to remove intact cells and nuclei, and the supernatants were further centrifuged at 8000*g* for 7 min to precipitate mitochondria. The mitochondrial pellet was extensively washed with buffer A and resuspended in buffer C (220 mM mannitol, 68 mM sucrose, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.4, 5 mM succinic acid, 5 mM ATP, 10 mM *b*-glycerophosphate, 10 μg/ml aprotinin, 0.25 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). After 30 min of stabilization at 37°C, mitochondria were resuspended in buffer C at the density of 1.0 mg of mitochondrial protein/ml and used for the cell-free assay. The concentration of mitochondrial proteins in the

supernatant was determined using a protein estimation kit (Bio-Rad). Isolated mitochondria (1.0 mg/ml) were preincubated with 0.15 μg of active or unactive ERK in 30 μl of buffer C for 1 h and incubated with 0.3 μg of active JNK for 2 h at 37°C. After centrifugation at 14,000*g* for 10 min to remove mitochondria, the supernatant and mitochondrial pellets were analyzed by Western blotting for the presence of cytochrome *c* and Bcl-2, respectively. Mitochondrial inner membrane potential was assessed by using DiOC6 and flow cytometry (Aoki et al., 2002; Castedo et al., 2002).

Results

PMA Inhibits MG-Induced Apoptosis. To evaluate the effect of PMA on MG-induced apoptosis, we first examined the effect of PMA on MG-induced DNA fragmentation. Treatment of Jurkat cells with 250 μM MG alone resulted in the induction of apoptosis as indicated by the increasing percentage of DNA fragmented cells in time-course analysis (Fig. 1B). When 50 ng/ml PMA was added 1 h before MG, apoptosis was completely inhibited (Fig. 1, A and B).

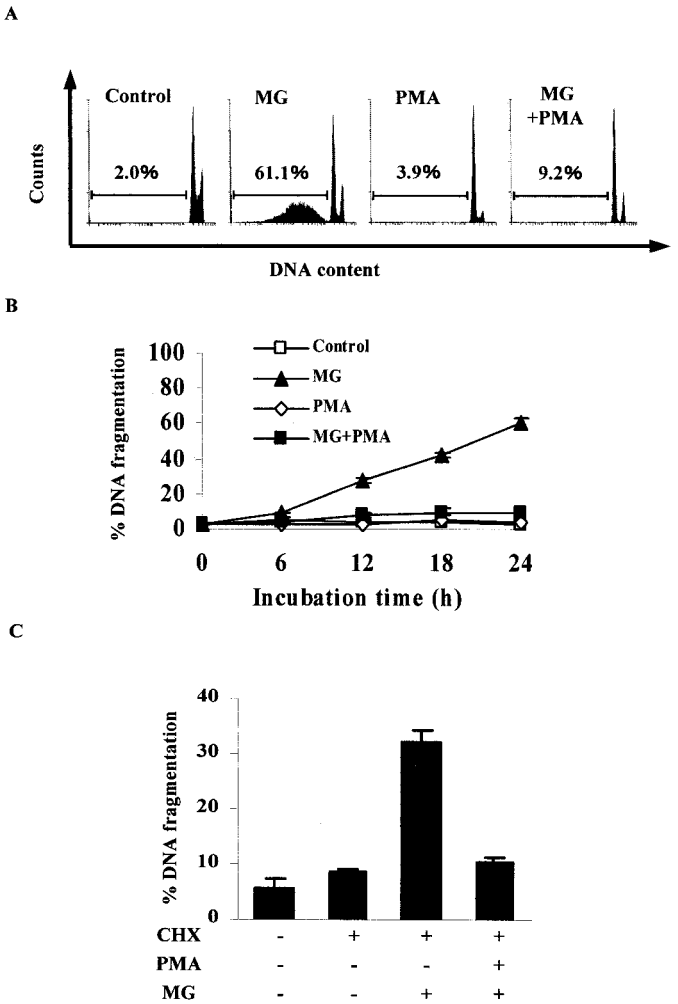

Fig. 1. PMA inhibits MG-induced apoptosis in Jurkat cells. A, Jurkat cells were preincubated with or without 50 ng/ml PMA for 1 h and treated with 250 μM MG. After 24 h of incubation, cells were analyzed by staining with PI and subjected to flow cytometry. The percentage of cells containing <2 N DNA is shown. B, Jurkat cells were exposed to a different effector as described in A for 0 to 24 h and analyzed for DNA fragmentation. C, Jurkat cells were preincubated with 1 μg/ml cycloheximide for 1 h and treated with 50 ng/ml PMA for 1 h. The cells were exposed to 250 μM MG for 9 h and analyzed for DNA fragmentation. Results are expressed as means ± S.D. from triplicate cultures.

We also determined the protein synthesis requirement for PMA-induced resistance. Jurkat cells were pretreated with 1 μg/ml cycloheximide (CHX) for 1 h and subsequently incubated for a short time (9 h) in the presence of MG. As shown in Fig. 1C, although CHX itself induced low levels of apoptosis, the PMA-induced survival effect was not disrupted by CHX treatment, indicating that PMA can protect MG-induced apoptosis without new protein synthesis.

Caspase-8 Is Not Necessary for MG-Induced Mitochondrial Dysfunction and Apoptosis. Several studies have shown that PMA can inhibit death receptor-mediated apoptosis by disrupting the process of death-inducing signaling complex assembly and subsequent caspase-8 activation (Gomez-Angelats and Cidlowski, 2001). Thus, we examined whether caspase-8 is important for the induction of apoptosis by MG. To evaluate the involvement of caspase-8 in MG-induced apoptosis, we used a caspase-8–negative Jurkat cell line, I9-2, and assessed apoptotic cells. As shown in Fig. 2A, there were no significant differences between the two cell lines. This implies that caspase-8 is not a crucial factor for MG-induced triggering of apoptosis, because another group has reported that an anti-Fas antibody could not induce apoptosis in I9-2 cells (Belka et al., 2000).

We further investigated the involvement of caspase-8 in the change in MG-induced mitochondrial membrane permeability (MMP). In our study, permeability of the outer membrane was assessed by detecting cytosolic cytochrome *c* using Western blotting, and depolarization of the inner membrane was assessed by measuring the ΔΨm breakdown by uptake of DiOC6, a cationic fluorochrome. To evaluate the effect of caspase-8 on MMP after MG treatment, cells were incubated with MG for 5 h, and cytochrome *c* release and ΔΨm were analyzed. As shown in Fig. 2, B and C, MG caused both cytochrome *c* release and ΔΨm decline even in the absence of caspase-8, implying that caspase-8 is not needed for the MMP change in MG-induced apoptosis. In addition, we examined whether PMA can protect I9-2 cells against MG-induced apoptosis. As shown in Fig. 2D, PMA completely abrogated MG-induced apoptosis in I9–2 cells, as was the case in Jurkat cells (Fig. 1A). These results show that PMA can also inhibit caspase-8–independent apoptotic signals in Jurkat cells, suggesting the existence of intervening signal points by PMA that have not been identified yet (Gomez-Angelats and Cidlowski, 2001; Meng et al., 2002).

Antiapoptotic Effect of PMA Depends on the MEK/ERK Pathway. Previous studies have demonstrated that PMA efficiently activates the MEK/ERK pathway in lymphoid cells (Li et al., 1999; Herrant et al., 2002; Meng et al., 2002). To confirm the possible role of this pathway in PMA-mediated cell survival, Jurkat cells were treated with PMA in the absence or presence of the MEK1 inhibitor PD98059. PD98059 clearly sensitized Jurkat cells to MG-induced apoptosis, whereas treatment with PD98059 by itself had no demonstrable effect on these cells (Fig. 3A). Moreover, the inhibition of apoptosis by PMA was almost completely prevented by PD98059. In addition, as shown in Fig. 3B, phosphorylation levels of MEK and ERK were significantly correlated with the ability of PMA to promote cell survival. These results suggest that the MEK/ERK pathway plays a pivotal role in PMA-mediated cell survival.

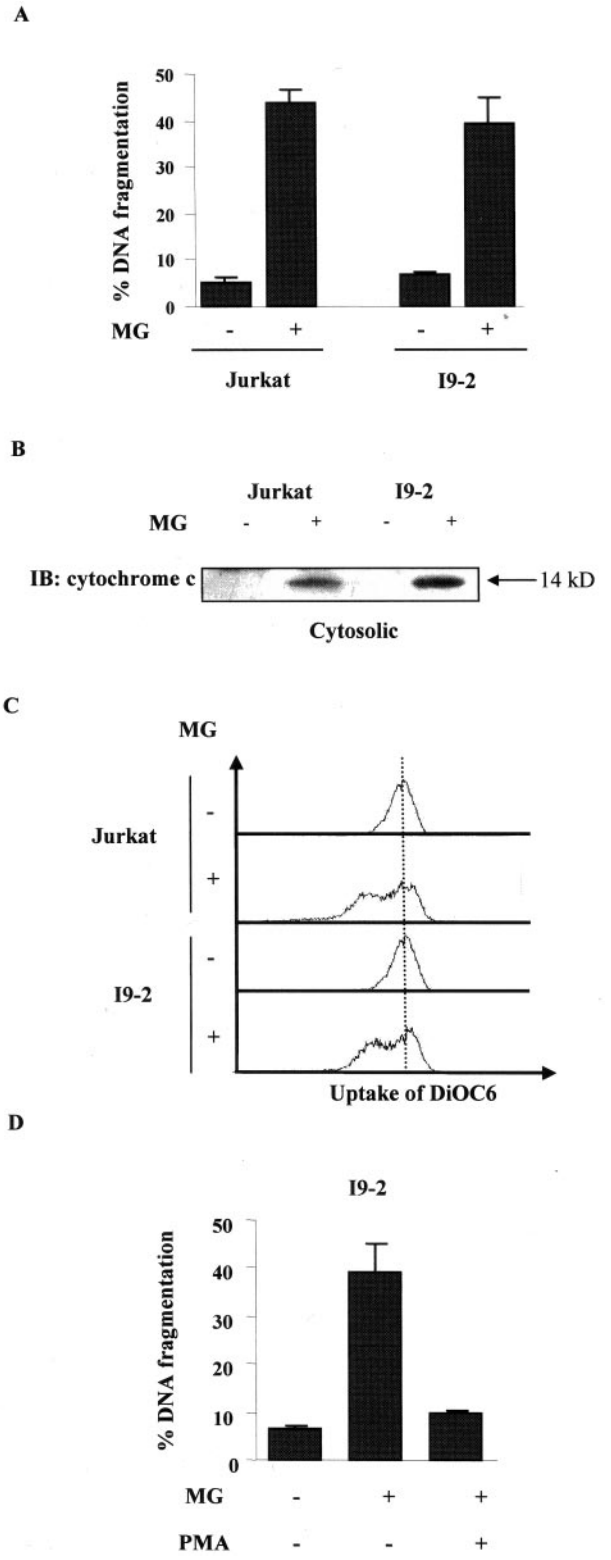

Fig. 2. Caspase-8 is not necessary for MG-induced mitochondrial dysfunction and apoptosis. A, Jurkat cells or I9–2 cells were treated with 250 μM MG for 20 h, and DNA fragmentation was analyzed by flow cytometry with PI. B and C, Jurkat cells or I9-2 cells were treated with 250 μM MG for 4 h. Cytosolic cytochrome *c* was determined by Western blot analysis, and mitochondrial membrane potential was measured by flow cytometry with DiOC6 dye. Results are representative of three independent experiments. D, I9-2 cells were preincubated with or without 50 ng/ml PMA for 1 h and treated with 250 μM MG for 18 h. The cells were analyzed by flow cytometry with PI staining. Results are expressed as means ± S.D. from triplicate cultures (A and D).

We further confirmed the importance of the MEK/ERK pathway by using dominant-negative mutants of Ras. In a previous study, Li et al. (1999) demonstrated that transfection of Jurkat cells with dominant-negative Ras N17 prevented PMA from stimulating the activity of ERK. Thus, we established cell lines constantly expressing the wild-type Ras (Ras WT) or dominant-negative Ras (Ras N17). The results presented in Fig. 3, C and D, show that dominant-negative Ras N17 mutants abrogated PMA-induced MEK activation. We next assessed the levels of apoptosis by stimulating three cell lines, Jurkat cells, Ras WT cells, and Ras N17 cells, with MG. As shown in Fig. 3E, there was no significant difference between the levels of apoptosis in the three cell lines when the cells were treated with MG alone. However, in the case of treatment with MG plus PMA, Ras N17 cells were not rescued from MG-induced apoptosis, although PMA completely prevented apoptosis in Jurkat cells and Ras WT cells. These results suggest that the Ras/MEK/ERK pathway plays a critical role in the inhibition of MG-induced apoptosis by PMA treatment.

The MEK/ERK Pathway Modulates Caspase Activation in MG-Treated Jurkat Cells. To determine whether caspase activation is functionally important for MG-induced apoptosis, Jurkat cells were coincubated with the pan-caspase inhibitor z-VAD-fmk, and then apoptotic cells were assessed. As shown in Fig. 4A, pretreatment with z-VAD-fmk completely blocked the MG-induced apoptosis. These results suggest that MG-induced apoptosis occurs through the activation of caspase(s). To understand the effect of the MEK/ERK pathway on caspase activation, we examined the effect of PD98059 on the cleavage of caspase-9 and -3. The data in Fig. 4B show that PMA prevents MG-induced caspase cleavage, and the MEK/ERK pathway mediates this effect. These results indicate that the MEK/ERK pathway regulates caspase activation and apoptosis in MG-stimulated Jurkat cells.

Caspase activation has been reported to act upstream and downstream of mitochondrial dysfunction (Creagh and Martin, 2003). Although we showed in Fig. 2 that caspase-8 is not necessarily needed for upstream regulator of mitochondria, it is not clear whether other member of caspases, including caspase-9 and caspase-3, act as a regulator of mitochondria. We therefore examined whether MG-induced cytochrome *c* release is a consequence of caspase activation. Jurkat cells were preincubated with z-VAD-fmk before MG exposure, and then we detected cytosolic cytochrome *c*. As shown in Fig. 4C, cotreatment with z-VAD-fmk did not prevent cytochrome *c* release, suggesting that MG-induced cytochrome *c* release is not a consequence of caspase activation in MG-induced apoptosis.

Activation of the MEK/ERK Pathway Abrogates the Release of Cytochrome *c* but Does not Modulate $\Delta\Psi$m in MG-Treated Cells. Figure 2, B and C, show that MG causes at least two major changes in mitochondria as early apoptotic events: cytochrome *c* release, and breakdown of $\Delta\Psi$m.

To evaluate the role of the MEK/ERK pathway in prevention of increase in MMP in MG-treated cells, we examined the levels of release of cytochrome *c* in Jurkat cells treated with MG in the presence and absence of PD98059. Figure 5A shows that PMA counteracted the MG-induced release of cytochrome *c* and that PD98059 reversed its protective effect.

These observations indicate that the MEK/ERK pathway regulates the permeability of the outer mitochondrial membrane and prevents the release of cytochrome *c* into the cytosol.

We next evaluated the influence of the MEK/ERK pathway on ΔΨm. Treatment with MG for 2.5 h induced a remarkable decline in ΔΨm, which was not observed in cells treated with PMA or PD98059 alone (Fig. 5B). Pretreatment with PMA before MG prevented the breakdown of ΔΨm in agreement with the results for cytochrome *c* release. Unexpectedly, however, combined pretreatment with PD98059 and PMA did not result in a remarkable decline in ΔΨm compared with that in the case of treatment with MG alone. To confirm the observed retention of ΔΨm in cells treated with PD98059, we conducted an additional experiment using another MEK inhibitor, U0126. In agreement with the results with PD98059, U0126 also failed to affect ΔΨm in MG-treated Jurkat cells. Thus, in our system, MEK inhibitors induced the release of cytochrome *c* but not a decline in ΔΨm under the condition of costimulation with MG and PMA. Therefore, our results suggest that extrinsic activation of the MEK/ERK pathway may prevent MG-induced release of cytochrome *c* by selective regulation of the outer membrane. The antiapoptotic effect of MEK/ERK pathway seems to act upstream or at the mitochondrial level.

PMA Does Not Down-Regulate MG-Induced Activation of the JNK Pathway. We previously demonstrated that MG rapidly increases ROS production and that ROS-triggered JNK activation contributes to the induction of apoptosis in MG-treated Jurkat cells (Du et al., 2000, 2001). To evaluate the possible effect of PMA on the JNK pathway, we carried out an experiment to determine whether MG-induced activation of the JNK pathway is influenced by pretreatment with PMA. Jurkat cells were treated with MG for 0 to 2 h in the presence or absence of PMA and were analyzed by immunoblotting with anti–phospho-MKK4 and -JNK antibodies. As shown in Fig. 6A, MG induced a rapid and strong activation of both MKK4 and JNK, but PMA did not decrease these phosphorylation levels. In contrast, the antioxidant NAC clearly reduced the level of MG-induced JNK activation and subsequent cell death (Fig. 6, B and C).

Translocation of JNK from cytosol to the nucleus has been shown in response to osmotic stress (Kawasaki et al., 1996). Other studies showed that JNK translocates to the mitochondria after genotoxic stress and inhibits the antiapoptotic function of proteins belonging to Bcl-2 family members (Kharbanda et al., 2000; Kroemer and Reed, 2000). We next examined subcellular distribution and activation of JNK in MG-treated Jurkat cells. Subcellular localization of JNK was assessed by subjecting cytosolic and mitochondrial fractions to immunoblotting with an anti-JNK antibody. As shown in Fig. 6D, MG induced phosphorylation of JNK in both fractions but did not significantly change the subcellular distribution of JNK. Pretreatment with PMA had no effect on localization and phosphorylation of JNK. These findings suggest that PMA does not inhibit MG-induced activation of JNK at the whole-cell level. Additionally, PMA also had no effect on either the redistribution or activation of JNK on mitochondria.

Active JNK Can Directly Induce the Release of Cytochrome *c* from Jurkat Cell-Derived Mitochondria. Active JNK has recently been shown to induce cytochrome *c* release from isolated mitochondria derived from cardiac myocytes and brain cells (Aoki et al., 2002; Schroeter et al., 2003). We therefore carried out an experiment to determine whether phosphorylated JNK also induces the release of

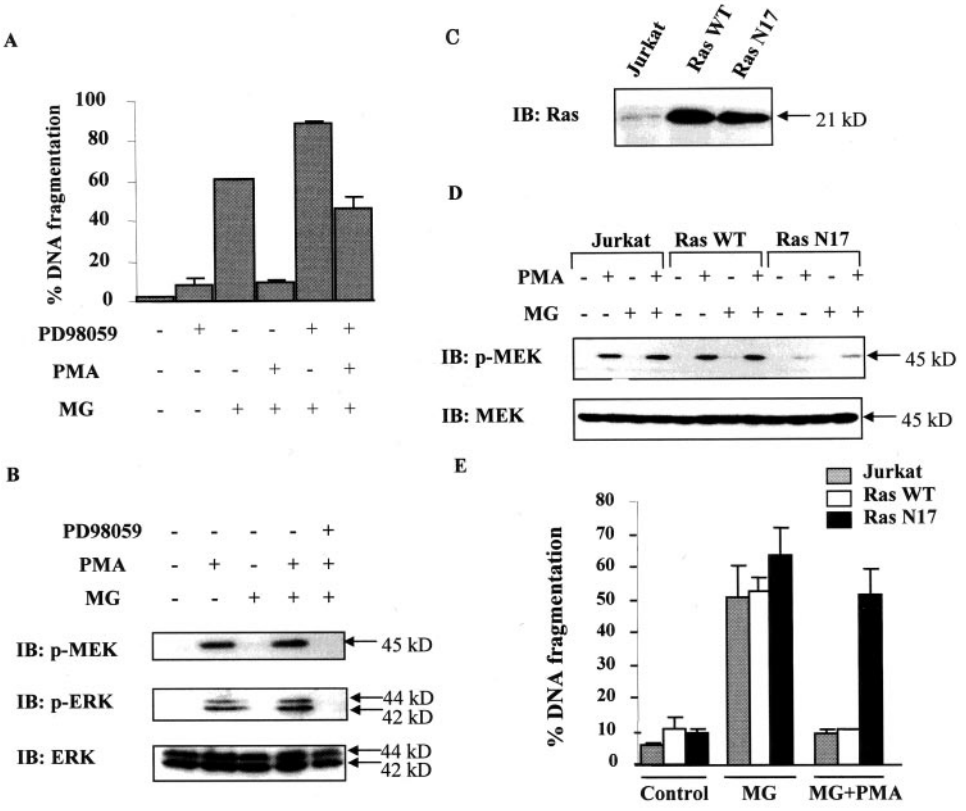

Fig. 3. Antiapoptotic effect of PMA depends on the MEK/ERK pathway. A, Jurkat cells were preincubated with or without 50 μM PD98059 for 1 h and then treated with 50 ng/ml PMA for 1 h. The cells were incubated with 250 μM MG for 20 h and then analyzed by flow cytometry with PI staining. B, Jurkat cells were preincubated with PMA and/or PD98059 as above and then exposed to 250 μM MG for 0.5 h. Activation of MEK and ERK was determined by Western blot analysis. C, Jurkat cells were stably transfected with wild-type Ras or a dominant-negative Ras vector. Expression of Ras was determined by Western blot analysis. D, Jurkat, Ras WT, and Ras N17 cells were preincubated with or without 50 ng/ml PMA for 1 h and then treated with 250 μM MG for 0.5 h. Activation of MEK was determined by Western blot analysis. E, Jurkat, Ras WT, and Ras N17 cells were preincubated with or without 50 ng/ml PMA for 1 h and then treated with 250 μM MG for 20 h. Cells were analyzed by flow cytometry staining with PI. The data represent means ± S.D. of triplicate cultures (A and E).

cytochrome *c* in mitochondria derived from Jurkat cells. Mitochondria were isolated from Jurkat cells by differential centrifugation and suspended in reaction buffer containing ATP. After incubation with a recombinant active JNK for 2 h, mitochondria were removed by centrifugation, and the supernatant obtained was analyzed for the presence of cytochrome *c* by Western blotting. As shown in Fig. 7A, JNK caused an observable release of cytochrome *c*, indicating that active JNK can induce permeabilization of the outer membrane. Treatment with JNK had no effect on the amount of Bcl-2, an antiapoptotic protein of mitochondria.

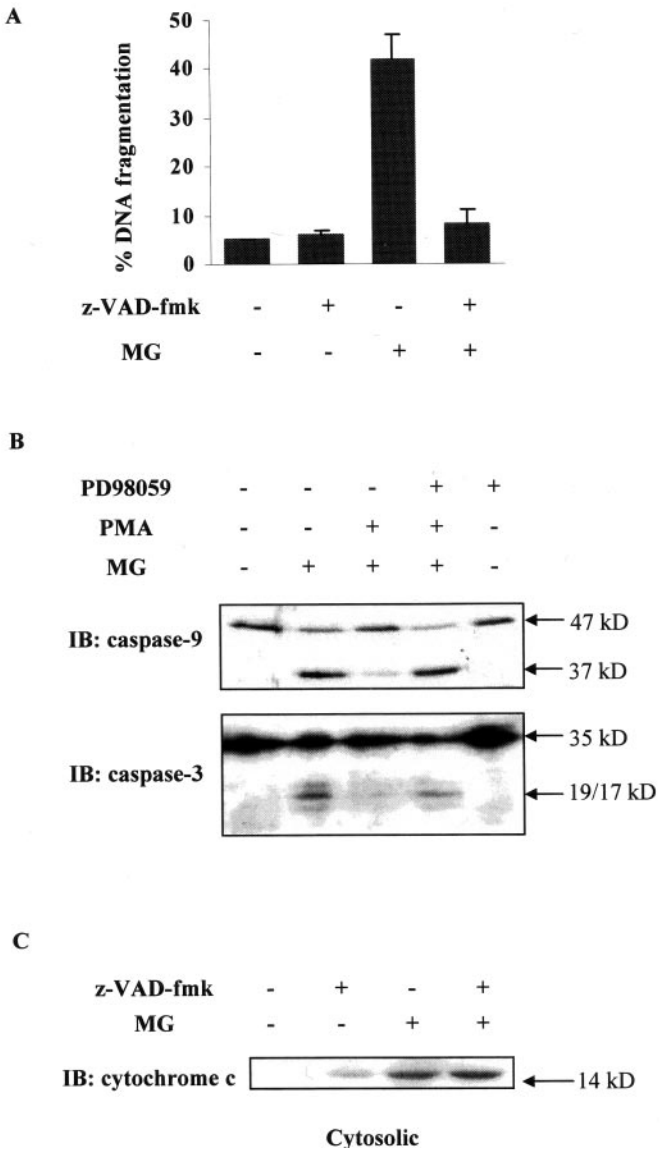

Fig. 4. The MEK/ERK pathway modulates caspase activation in MG-treated Jurkat cells. A, Jurkat cells were preincubated with or without 50 μM z-VAD-fmk for 1 h and then exposed to 250 μM MG for 18 h. The cells were analyzed by flow cytometry with PI staining. Results are expressed as means ± S.D. of triplicate cultures. B, Jurkat cells were preincubated with or without 50 μM PD98059 for 1 h and then treated with 50 ng/ml PMA for 1 h. The cells were exposed to 250 μM MG for 4 h. Activation of caspase-9 and -3 was determined by Western blot analysis. Results are representative of three independent experiments. C, Jurkat cells were preincubated with or without z-VAD-fmk as above and then exposed to 250 μM MG for 4 h. After preparation of the subcellular fraction, cytosolic cytochrome *c* was detected by Western blot analysis. Results are representative of three independent experiments.

Next, we assessed $\Delta\Psi$m of JNK-treated mitochondria by flow cytometric analysis of DiOC6 uptake. Treatment of isolated mitochondria with JNK did not cause detectable depolarization compared with that in the case of treatment with valinomycin (Fig. 7B). It is known that enhancement of outer membrane permeability, even in the absence of elevation of inner membrane permeability, results in the release of cytochrome *c* from mitochondria (Newmeyer and Ferguson-Miller, 2003). Thus, our results suggest that active JNK causes an increase in permeabilization of the outer membrane without depolarization of the inner membrane. Our results agree with results reported previously from a study using mitochondria from cardiac myocytes (Aoki et al., 2002). Intriguingly, because the expression of a dominant-negative mutant of JNK reduces the level of $\Delta\Psi$m in MG-treated Jurkat cells (Du et al., 2000), our in vitro results presented here indicate that, at the whole-cell level, JNK regulates $\Delta\Psi$m probably in cooperation with other unidentified factors that are present in cytosol or by an indirect mechanism that does not accompany its direct action.

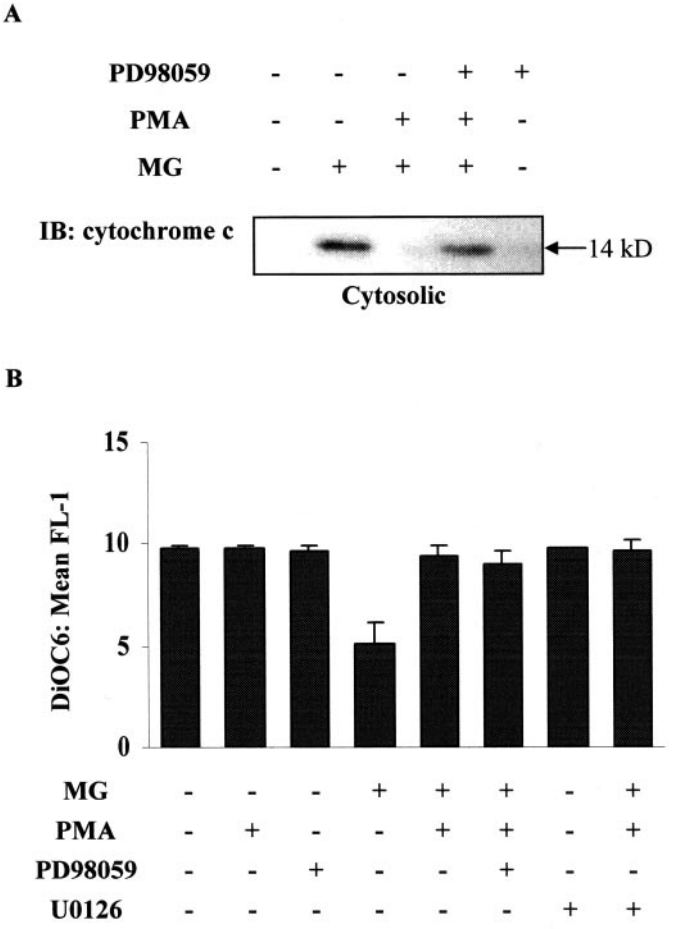

Fig. 5. MEK/ERK pathway abrogates the release of cytochrome *c* but does not regulate mitochondrial membrane potential in MG-induced apoptosis. A, Jurkat cells were preincubated with or without 50 μM PD98059 for 1 h and then treated with 50 ng/ml PMA for 1 h. The cells were exposed to 250 μM MG for 4 h. After preparation of the subcellular fraction, cytosolic cytochrome *c* was detected by Western blot analysis using the cytosolic fraction. B, Jurkat cells were preincubated with PMA and/or PD98059 as above and then exposed to 250 μM MG for 2.5 h. Alteration in mitochondrial membrane potential was measured by flow cytometry with DiOC6 staining. DiOC6 staining was quantified as mean FL-1 channel emission by flow cytometry. Data are expressed as means ± S.D. of four determinations.

Active ERK Inhibits JNK-Induced Release of Cytochrome *c*. It is possible that the PMA-mediated antiapoptotic effect acts on mitochondria to prevent JNK-induced apoptotic signaling. Recently, Baines et al. (2002) demonstrated that ERK is localized in cardiac mitochondria and that transgenic activation of PKCε enhances mitochondrial ERK phosphorylation compared with nontransgenic mitochondria. Thus, we investigated the effect of PMA on the phosphorylation of mitochondrial ERK. As shown in Fig. 8A, phosphorylation of ERK was detected in both fractions from cells treated with MG in the presence of PMA, whereas treatment with MG alone had no effect on localization and activation of ERK. These findings raise the possibility that the MEK/ERK pathway is linked to mitochondrial death machinery and inhibits JNK-induced apoptotic signaling.

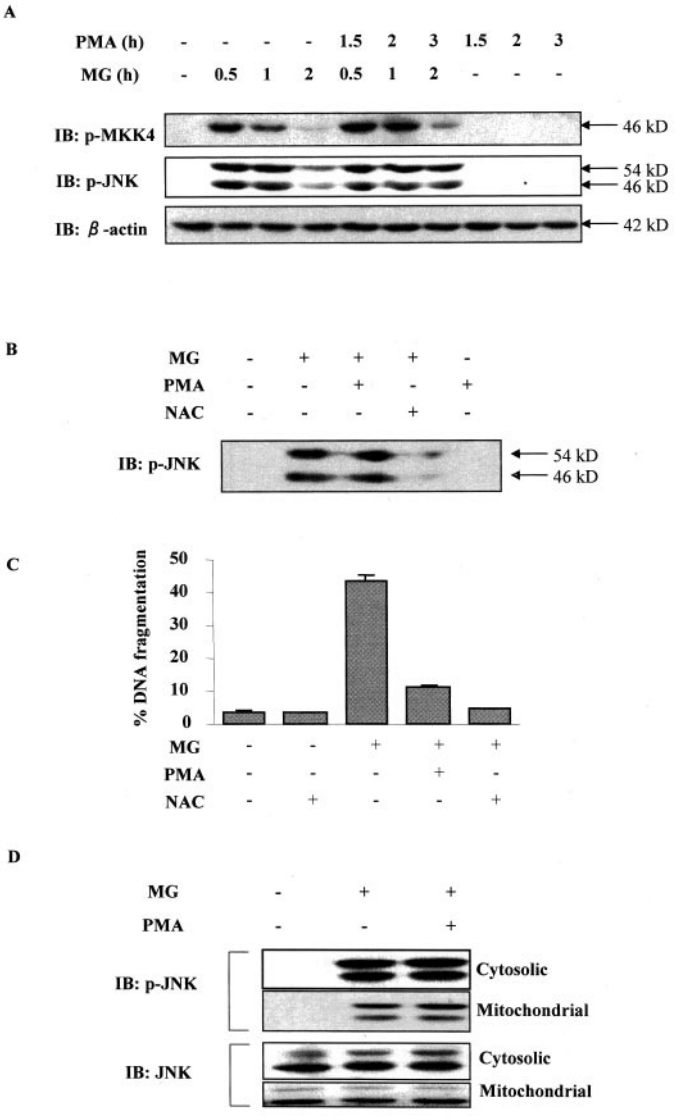

Fig. 6. PMA does not down-regulate MG-induced JNK activation. A, Jurkat cells were preincubated with or without 50 ng/ml PMA for 1 h and then treated with 250 μM MG for the indicated time periods. Activation of JNK and MKK4 was determined by Western blot analysis. B, Jurkat cells were preincubated with 50 ng/ml PMA or 20 mM NAC for 1 h and then treated with 250 μM MG for 0.5 h. Activation of JNK was determined by Western blot analysis. C, Jurkat cells were preincubated with PMA or NAC as described above and then treated with 250 μM MG for 20 h. The cells were analyzed by flow cytometry with PI staining. Results are expressed as means ± S.D. from triplicate cultures. D, Jurkat cells were preincubated with or without 50 ng/ml PMA for 1 h and then treated with 250 μM MG for 0.5 h. The cells were fractionated into cytosol and mitochondrial extracts, and each fraction was subjected to immunoblot analysis using anti–phospho-JNK or anti-JNK antibody. Results are representative of three independent experiments.

We also examined the effect of ERK on JNK-induced release of cytochrome *c* in a cell-free system. Mitochondria from Jurkat cells were preincubated with active ERK or unactive ERK for 1 h before treatment with JNK, and release of cytochrome *c* from mitochondria was demonstrated. The results showed that active ERK suppressed the redistribution of cytochrome *c* to the supernatant, as indicated by a decreased band (Fig. 8B). On the other hand, unactive ERK failed to suppress cytochrome *c* release, suggesting that ERK may regulate JNK-induced cytochrome *c* release in a kinase activity-dependent manner. The total amount of Bcl-2 in the mitochondrial pellet was not altered by treatment with JNK or ERK. In addition, we did not detect a migration shift of Bcl-2 induced by JNK or ERK in our experiment, although several groups have reported that JNK or ERK catalytically phosphorylates Bcl-2 protein in vitro and causes migration shift of Bcl-2 on SDS-polyacrylamide gel electrophoresis (Yamamoto et al., 1999).

PMA Treatment Prevents Murine Splenocytes from MG-Induced Apoptosis. To evaluate whether the cytoprotective effect of PMA is a characteristic unique to Jurkat cells, we examined the effect of PMA against MG-induced apoptosis using murine splenocytes. Our results show that murine splenocytes undergo apoptosis after exposure by MG (Fig. 9). PMA treatment, however, rescues splenocytes from MG-induced apoptosis. Inhibition of the MEK/ERK pathway by PD98059 reverses the cytoprotective effect of PMA, suggesting that activation of MEK/ERK pathway is critical for maintaining unresponsiveness to MG-induced apoptosis in murine splenocytes. Our results using murine splenocytes show that the cytoprotective effect of PMA is not a unique characteristic to Jurkat leukemia cell line but is shared by normal cells.

Discussion

In this study, we demonstrated a protective function of ERK in apoptotic events trigged by the physiological stress factor methylglyoxal. Activation of the ERK pathway after PMA treatment resulted in the inhibition of mitochondrial death machinery induced by exposure to MG. Because MG caused JNK activation and activated JNK was sufficient for inducing cytochrome *c* release from mitochondria, we speculated that ERK is involved in the regulation of JNK-mediated mitochondrial dysfunction. In support of this hypothesis, the ERK and the JNK signals, which are triggered by PMA and MG, respectively, were integrated on mitochondria. We further demonstrated that active ERK represses JNK-induced leakage of cytochrome *c* in vitro. Because the protective effect of ERK on JNK-induced mitochondrial death machinery has not been demonstrated directly in previous studies, our study is the first in which direct evidence of their opposing effects on mitochondrial injury was obtained using a cell-free system.

Our experimental model is different from other systems that were designed to investigate the effects of PMA on death-receptor signaling. A number of previous studies have

demonstrated that PMA inhibits caspase-8 activation in Fas-mediated death signals and prevents consequent cell death (Meng et al., 2002; Engedal and Blomhoff, 2003). Because caspase-8 is a crucial factor for Fas-mediated apoptosis (Belka et al., 2000), inhibition of the caspase-8 pathway by PMA would be a favorable action that accounts for the resistance to apoptosis. However, in the case of the MG system, caspase-8 is not important for the initiation of apoptotic events involving mitochondrial dysfunction (Fig. 2). Thus, our experimental system revealed that PMA-mediated signals can also negatively regulate caspase-8–independent apoptotic events. In our additional search for other targets that induce cytochrome *c* release, we studied the Bid cleavage and the translocation of Bax to mitochondria. In our system, both Bid cleavage and Bax translocation have not initiated until after 8 h of MG exposure, whereas cytochrome *c* release was detected after 2 h (data not shown). These observations suggest that PMA can negatively regulate mitochondria-mediated cell death by MG independently of Bid cleavage and Bax translocation.

Moreover, our results here showed that PMA can prevent apoptosis through post-translational modification. PMA induces protein synthesis in Jurkat cells through activation of Raf/MEK/ERK modules. The ERK pathway activates cAMP response element-binding protein and nuclear factor-κB transcription factors (Ballif and Blenis, 2001), which upregulate the pro-survival Bcl-2 family members Bcl-2, Bcl-XL, and Mcl-1 and then promote cell survival (Ballif and Blenis, 2001). However, in our model, PMA treatment before MG challenge was relatively short, and PMA did not require new protein synthesis for protection, as indicated by the results of cycloheximide treatment (Fig. 1C). Therefore, our results indicate that PMA prevents MG-induced apoptosis through post-translational modification.

It has been shown in human endothelial cells and rat mesangial cells that activation of p38 by MG is linked to the induction of apoptosis (Akhand et al., 2001; Liu et al., 2003). Our preliminary experiment showed that in Jurkat cells MG also caused p38 phosphorylation strongly, but a specific inhibitor, p38, had no effect on MG-induced apoptosis. These observations indicate that in Jurkat cells, p38 is not functionally important for MG-induced apoptosis. On the other hand, intense activation of the JNK pathway is a critical trigger of apoptosis in our system (Du et al., 2000, 2001). We therefore carried out an experiment to determine whether PMA reduces phosphorylation levels of the JNK pathway under the condition of MG treatment. Although antioxidant NAC significantly abolished MG-induced JNK activation accompanying the depletion of intracellular ROS (Du et al., 2001), PMA failed to reduce phosphorylation levels of MKK4 and JNK (Fig. 6, A and C). Several groups have reported that growth factor-induced Akt activation negatively regulates the JNK signaling pathway and JNK-mediated apoptosis (Kim et al., 2001; Park et al., 2002). According to those reports, activated Akt phosphorylates and inhibits ASK1 and MKK4 in the JNK-signaling cascade and consequently attenuates JNK activation. However, in our system, PMA failed to counteract the activation of the JNK pathway. This may be because PMA, unlike growth factors, can not significantly activate Akt in Jurkat cells (Herrant et al., 2002; Engedal and Blomhoff, 2003). Collectively, our results suggest that PMA-induced protection of apoptosis may be caused by mechanisms different from those of NAC (via elimination of oxidative stress) and growth factors (via activation of Akt pathway). Considering that phosphorylation levels of mitochondrial JNK were unchanged by PMA treatment (Fig. 6D), PMA may exert an antiapoptotic effect downstream of JNK activation. Moreover, because PMA completely blocked mitochondrial dysfunction (Fig. 5A, B), PMA may suppress the JNK-mediated action at the level of mitochondria by preventing the release of apoptosis-promoting factors such as cytochrome *c*.

Data obtained using inhibitors indicate that the MEK/ERK pathway is required for the protection of the release of cytochrome *c* and activation of caspases (Figs. 4B and 5A). These results suggest that the MEK/ERK pathway regulates mitochondrial death machinery by modulating the outer mitochondrial membrane. Consistent with in vivo results, recombinant ERK prevented JNK-induced leakage of cytochrome *c*, which occurs with permeabilization of the outer membrane (Figs. 7 and 8).

It is obviously important to clarify how ERK inhibits the release of cytochrome *c*. Several groups have reported that ERK can phosphorylate mitochondrial protein directly or indirectly. First, Bad was found to be phosphorylated at the site of serine 112 through ERK-mediated activation of p90Rsk (Scheid et al., 1999). Second, Bcl-2 also seems to be a target of ERK, and ERK-induced phosphorylation of Bcl-2

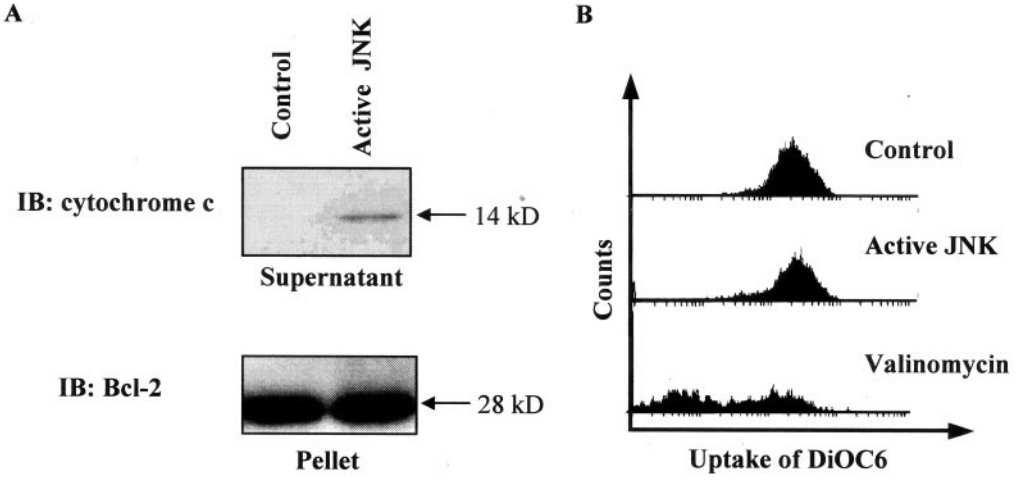

Fig. 7. Active JNK can directly induce the release of cytochrome *c* from Jurkat cell-derived mitochondria. A, mitochondria isolated from Jurkat cells were incubated for 2 h with 0.3 μg of active JNK at 37°C. Reaction supernatants and mitochondrial lysates were immunoblotted with anti-cytochrome *c* and anti-Bcl-2 antibodies, respectively. B, mitochondria were treated with JNK or 1 μM valinomycin for 2 h. Mitochondrial inner membrane potential was analyzed by flow cytometry with DiOC6 staining. Results are representative of three independent experiments.

probably enhances its antiapoptotic effect, probably through stabilization of Bcl-2-Bax heterodimerization (Deng et al., 2000). Moreover, it has recently been reported that IEX-1, a novel substrate of ERK, is localized mainly in mitochondria and protects different cell types against various apoptotic triggers (Garcia et al., 2002). However, the molecular action downstream of JNK and ERK is not clear, because intervening molecules between JNK and mitochondria have not been determined (Aoki et al., 2002). Putative cross-talk between ERK and JNK on mitochondria needs to be addressed by further experiments.

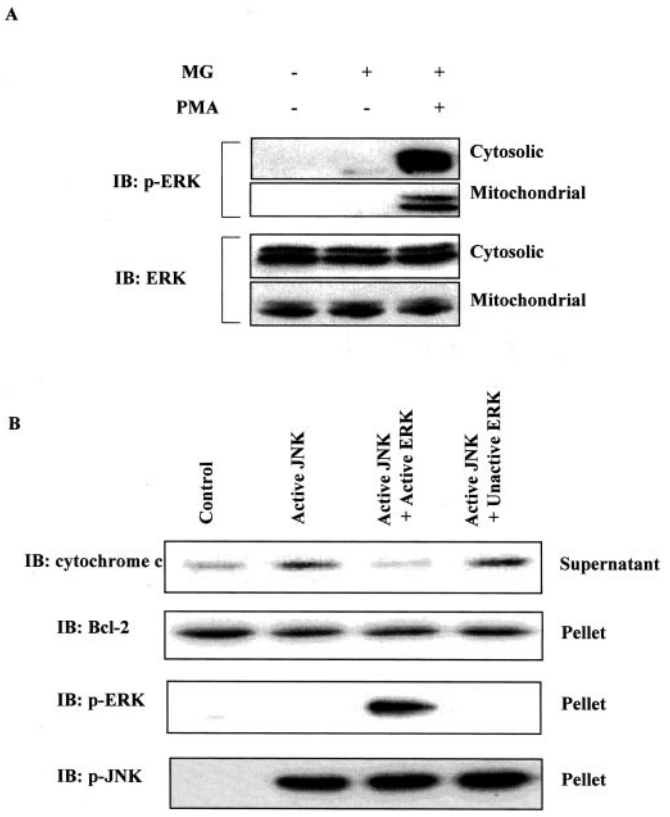

Fig. 8. Active ERK inhibits the JNK-induced release of cytochrome *c*. A, Jurkat cells were preincubated with or without 50 ng/ml PMA for 1 h and then treated with 250 μM MG for 0.5 h. The cells were fractionated into cytosol and mitochondrial extracts, and each fraction was subjected to immunoblotting analysis using anti–phospho-ERK or anti-ERK antibody. B, isolated mitochondria were preincubated with 0.15 μg of active or inactive ERK for 1 h and then treated with 0.3 μg of active JNK for 2 h at 37°C. After centrifugation, the supernatant was analyzed for the presence of cytochrome *c* by Western blotting. The mitochondrial fraction was immunoblotted with anti-Bcl-2, anti–phospho-ERK, and anti–phospho-JNK antibodies. Results are representative of three independent experiments.

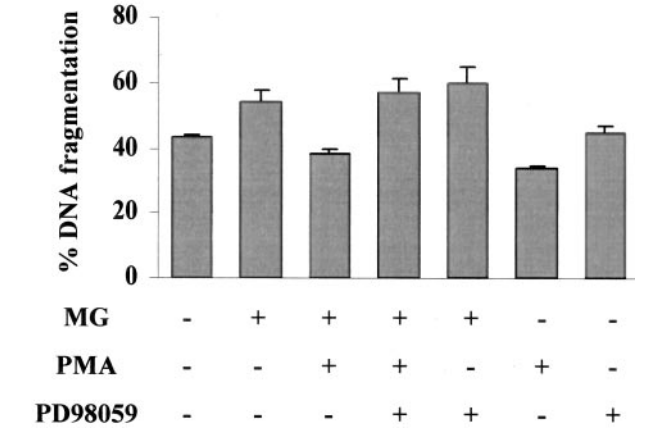

Fig. 9. PMA treatment prevents murine splenocytes from MG-induced apoptosis. Splenocytes were preincubated with or without 30 μM PD98059 for 1 h and then treated with 20 ng/ml PMA for 30 min. The cells were incubated with 50 μM MG for 6 h and then analyzed by flow cytometry with PI staining. Data are presented as the mean ± S.D. of triplicate determinations.

Our results showed that neither of the MEK inhibitors PD98059 and U0126 reversed PMA-mediated retention of $\Delta\Psi$m in MG-treated Jurkat cells, indicating that activation of the MEK/ERK pathway by PMA is probably not involved in the retention of $\Delta\Psi$m (Fig. 5). Consistent with our findings, Yu et al. (2001) have reported that MEK inhibitors promoted the release of cytochrome *c* and apoptosis but not $\Delta\Psi$m decline in HL-60 cells treated with 1-β-D-arabinofuranosylcytosine, an anti-tumor agent. Several groups have reported that induction of apoptosis and cytochrome *c* release can be dissociated from $\Delta\Psi$m decline in some systems (Newmeyer and Ferguson-Miller, 2003). In some cases, permeability of each membrane, inner and outer, may be regulated separately by independent mechanisms (Newmeyer and Ferguson-Miller, 2003). The question then arises as to how PMA protects MG-induced $\Delta\Psi$m decline in Jurkat cells. Several molecules have been reported to act as antiapoptotic factors. With regard to PMA, responsible molecules, PKCα, PKCϵ, and Raf, have been reported to be regulators of mitochondrial protein and serve as antiapoptotic signals (Ruvolo et al., 1998; Chen et al., 2001; Wang et al., 2002). Although the effect of these molecules on $\Delta\Psi$m remains unknown, our findings suggest that these molecules have the ability to regulate $\Delta\Psi$m under the condition of PMA treatment. Taken together, our results provide a unique model of regulation of MMP by PMA in MG-treated Jurkat cells. That is, under the condition of PMA stimulation, 1) permeability of the outer membrane, the enhancement of which leads to cytochrome *c* release, is conserved by at least the MEK/ERK pathway, and 2) permeability of the inner membrane, the enhancement of which leads to a decline in $\Delta\Psi$m, is conserved by PMA-sensitive but MEK-independent signaling pathways.

In summary, we have found that ERK activation by PMA contributes to the protection of MG-induced apoptosis in Jurkat cells. PMA prevented mitochondrial damage, including the loss of $\Delta\Psi$m and cytochrome *c* release. In an attempt to further clarify the relationship between ERK and JNK, we found that ERK can counteract JNK-induced leakage of cytochrome *c* from mitochondria, thus leading to the prevention of subsequent mitochondrial apoptotic events. In this study, we used PMA as a model agent that activates the MEK/ERK pathway. However, PMA may not be used as clinical drug for intervening in apoptosis because of its adverse effects, such as the promotion of tumor (Liu and Heckman, 1998). Thus, further studies are needed to explore the alternative agents that selectively mimic the cytoprotective effect of PMA.

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